

# Photoinduced Removal of Nifedipine Reveals Mechanisms of Calcium Antagonist Action on Single Heart Cells

ALISON M. GURNEY, JEANNE M. NERBONNE, and  
HENRY A. LESTER

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

**ABSTRACT** The currents through voltage-activated calcium channels in heart cell membranes are suppressed by dihydropyridine calcium antagonists such as nifedipine. Nifedipine is photolabile, and the reduction of current amplitude by this drug can be reversed within a few milliseconds after a 1-ms light flash. The blockade by nifedipine and its removal by flashes were studied in isolated myocytes from neonatal rat heart using the whole-cell clamp method. The results suggest that nifedipine interacts with closed, open, and inactivated calcium channels. It is likely that at the normal resting potential of cardiac cells, the suppression of current amplitude arises because nifedipine binds to and stabilizes channels in the resting, closed state. Inhibition is enhanced at depolarized membrane potentials, where interaction with inactivated channels may also become important. Additional block of open channels is suggested when currents are carried by  $\text{Ba}^{2+}$  but is not indicated with  $\text{Ca}^{2+}$  currents. Numerical simulations reproduce the experimental observations with molecular dissociation constants on the order of  $10^{-7}$  M for closed and open channels and  $10^{-8}$  M for inactivated channels.

## INTRODUCTION

Voltage-activated calcium channels are present in many cell types and are generally thought to play important physiological roles. In cardiac and smooth muscle, for example, these channels provide an important link in excitation-contraction coupling. Organic "calcium antagonists" or "calcium entry blockers" inhibit ion flow through these channels (Fleckenstein, 1977, 1983; Nayler, 1983; Janis and Triggle, 1983; Smith, 1983) and are effective in the treatment of cardiovascular disorders such as angina, arrhythmias, and hypertension (Nayler, 1983). Although frequently grouped together and described collectively, " $\text{Ca}^{2+}$  antagonists" have diverse structures, a fact that suggests that more than one site and/or mechanism of action may be responsible for their various effects.

Address reprint requests to Dr. Alison M. Gurney, Dept. of Pharmacology, St. Thomas's Hospital Medical School, London SE1 7EH, U.K. Dr. Nerbonne's present address is Dept. of Pharmacology, Washington University School of Medicine, St. Louis, MO 63110.

The  $\text{Ca}^{2+}$  antagonists verapamil, D600, and diltiazem display strong use- or frequency-dependent blockade of calcium currents (Ehara and Kaufmann, 1978; McDonald et al., 1980; Lee and Tsien, 1983), which is easily observed under voltage-clamp conditions. Bath application of low concentrations of D600, for instance, has little effect on the amplitudes of  $\text{Ca}^{2+}$  currents in the absence of repetitive depolarization (Lee and Tsien, 1983). Such observations suggest that these compounds act by preferentially blocking open (rather than closed)  $\text{Ca}^{2+}$  channels and, therefore, that they behave as classical "open channel blocking" drugs (Armstrong, 1966; Strichartz, 1973; Adams, 1976). In addition, verapamil and diltiazem appear to have a higher affinity for inactivated calcium channels and display a greater degree of steady state block at depolarized potentials (McDonald et al., 1980; Kanaya et al., 1983). All of these properties are consistent with the "modulated receptor" hypothesis of drug action (Hille, 1977; Hondeghem and Katzung, 1977), in which the affinity of the drug for the receptor is modulated by the kinetic state of the channel (Hondeghem and Katzung, 1984).

Dihydropyridine derivatives, exemplified by nitrendipine and nifedipine, may act differently from other  $\text{Ca}^{2+}$  antagonists. Biochemical studies, for example, suggest that although all dihydropyridines apparently bind at the same site, this site is distinct from the one at which verapamil and diltiazem bind (DePover et al., 1982; Yamamura et al., 1982; Murphy et al., 1983; Holck et al., 1983). Early studies suggested that these compounds reduce the amplitude of the slow inward  $\text{Ca}^{2+}$  current ( $I_{\text{si}}$ ), primarily through interactions with resting (closed)  $\text{Ca}^{2+}$  channels (Lee and Tsien, 1983; Nerbonne et al., 1985; Uehara and Hume, 1984). Nevertheless, dihydropyridines appear to have some properties in common with other  $\text{Ca}^{2+}$  antagonists. In some cases, for example, it has been suggested that at least some of the blocking effect can be ascribed to an interaction with active (open) channels since dihydropyridines hasten the decay of  $I_{\text{si}}$  and display some use dependence when currents are evoked in rapid succession (Lee and Tsien, 1983; Sanguinetti and Kass, 1984b). The dihydropyridines do not, however, exhibit the slowly developing use-dependent block that is typical of other calcium antagonists (Lee and Tsien, 1983). Recent studies showed that a greater degree of inhibition of  $I_{\text{si}}$  is realized at depolarized membrane potentials; it has therefore been suggested that dihydropyridines also bind to inactivated channels (Bean, 1984; Sanguinetti and Kass, 1984b). Although dihydropyridines have recently been the subject of much interest, the relative importance of these various effects to the overall mechanism of action remains unclear.

Mechanistic studies of the  $\text{Ca}^{2+}$  antagonist effects of dihydropyridines and other  $\text{Ca}^{2+}$  antagonists are complicated by the fact that reversible blockade can only be effected after prolonged periods of washout. As a result, drug-induced suppression of  $I_{\text{si}}$  is not always clearly distinguishable from  $\text{Ca}^{2+}$  current rundown (Kostyuk, 1981). Nifedipine, like some other dihydropyridines, contains an *o*-nitrobenzyl moiety and is photolabile (Ebel et al., 1978); the reactions leading to photoconversion are complete within 100  $\mu\text{s}$  (Morad et al., 1983). Irradiation results in the destruction of nifedipine simultaneously with the production of a molecule devoid of  $\text{Ca}^{2+}$  antagonist activity (Ebel et al., 1978; Morad et al., 1983;

Sanguinetti and Kass, 1984a; Nerbonne et al., 1985). In frog atrial fibers, it is possible to produce nearly complete reversal of the suppression of  $I_{si}$  within, at most, a few milliseconds after a single light flash (1 ms duration) in the presence of  $\leq 1 \mu\text{M}$  nifedipine; at higher concentrations, recovery is similarly fast, although incomplete (Nerbonne et al., 1985). Irradiation of nifedipine, therefore, effectively makes it a rapidly reversible antagonist.

In the present experiments, we studied the blockade by nifedipine, and its removal by flashes, of whole-cell  $\text{Ca}^{2+}$  channel currents recorded from neonatal rat ventricular myocytes. The aim was to gain a better understanding of the mechanisms by which nifedipine reduces  $I_{si}$  and to determine the relative contributions made by interactions with the  $\text{Ca}^{2+}$  channel in its various states. The results suggest that when the inward current is carried by  $\text{Ba}^{2+}$ , nifedipine suppresses  $I_{si}$  through interactions with  $\text{Ca}^{2+}$  channels in all three kinetic states: the resting (closed), the activated (open), and the inactivated states. These findings are clearly consistent with the modulated receptor hypothesis of drug action (Hille, 1977; Hondgehem and Katzung, 1977, 1984). We suggest that at the normal resting potential of the cardiac cell, the main action of nifedipine is probably to block closed  $\text{Ca}^{2+}$  channels. At more depolarized potentials, however (for instance, when the cell is damaged), blockade of inactivated channels may become important.

A preliminary account of this work has previously appeared in abstract form (Gurney et al., 1984).

#### METHODS

Experiments were performed on individual cultured ventricular myocytes from neonatal rat hearts. The methods used in the isolation and preparation of cells were essentially the same as those described previously by Reuter et al. (1983). Cells, suspended in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, were plated at low density ( $\sim 1 \times 10^5$  cells/ml) on collagen-coated glass coverslips in 35-mm tissue culture dishes and maintained at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$ /95%  $\text{O}_2$  incubator at 100% relative humidity. Cells were used within 1–3 d of plating since, after this time, the cultures became overgrown with fibroblasts and heart cells were larger, factors that made it difficult to find individual myocytes that had clearly not fused with neighboring cells. For experiments, a coverslip with cells attached was transferred to the recording chamber, which contained serum-free physiological saline at  $20^\circ\text{C}$ , and was mounted on the stage of an inverted microscope (Diavert, E. Leitz, Inc., Rockleigh, NJ).

The whole-cell recording technique, first described by Hamill et al. (1981), was used to record ionic currents. The voltage-clamp circuit was provided by a patch clamp/whole-cell clamp (model 8900, DAGAN Corp., Minneapolis, MN) with a  $1\text{-G}\Omega$  feedback resistor. The slow inward current ( $I_{si}$ ) was separated in most experiments from overlapping outward  $\text{K}^+$  currents ( $I_K$ ) by using pipettes filled with  $\text{CsCl}$  (140 mM);  $\text{Cs}^+$ , apparently through dialysis with the intracellular medium, effectively blocks outward currents. The fast inward sodium current ( $I_{Na}$ ) was partially suppressed by the addition of  $20 \mu\text{M}$  tetrodotoxin (TTX) to the bathing solution; complete inactivation of  $I_{Na}$  was provided in most cases by holding the cell at  $-50 \text{ mV}$ . Under these conditions, uncontaminated recordings of  $I_{si}$  were obtained. Series resistance compensation was used and checked at regular intervals during each experiment. As previously described (Kostyuk, 1981), the current amplitude gradually declined during the recording sessions. It was usually possible, however, to record currents for 20–40 min after rupturing the membrane under the patch pipette.

### *Data Collection and Analysis*

$I_{si}$  was evoked by depolarizing voltage steps from holding potentials more negative than  $-40$  mV; experiments were usually conducted at  $-50$  mV. Linear leakage currents were subtracted before displaying and storing the data. All experimental parameters, e.g., holding potential, test potentials, flashlamp trigger, and the duration and timing of experimental trials, were controlled with an IBM personal computer equipped with a Labmaster analog interface (model 20009, Tecmar Corp., Cleveland, OH). Current signals were filtered at 3 kHz, digitized, and stored directly in digital form. The same computer was used later for data analysis (Kegel et al., 1985).

Data are expressed as means  $\pm$  SEM. Least-squares fits to the experimental data were made using the algorithm of Marquardt (1963), a computer program for which is described in Bevington (1969).

### *Optics*

The optical arrangement was essentially the same as that previously described (Nargeot et al., 1982). Flashes from a xenon short-arc flashlamp (Chadwick-Helmuth, Monrovia, CA) were filtered to remove wavelengths of  $<300$  nm (WG295, Schott Optical Glass, Inc., Duryea, PA); single flashes were produced by discharging the capacitor bank (2.1 mF) at 400 V. Alternative filters could readily be replaced in the optical path; similar results were obtained here using a variety of cut-off (to 335 nm) filters. Each flash delivered  $\sim 1$  J total output energy to the preparation; the flash duration was 1 ms. Light was usually presented to the preparation by mounting the lamp above the microscope stage, in the usual position of the illuminator, with a quartz condenser and focusing lens. Alternatively, flashes could be delivered through the microscope objective. Although the latter approach has the advantage of a small spot size, and therefore illumination of only a small portion of the bath, the former approach provides for higher energy output at wavelengths between 300 and 400 nm, the most useful wavelengths for photoconversion of nifedipine. Both methods were used in the experiments described here and gave consistent results.

In these experiments, we did not observe any of the nonspecific flash-induced artifacts previously noted with cardiac muscle strips (Nargeot et al., 1982, 1983; Morad et al., 1983; Sanguinetti and Kass, 1984a). In the absence of any photolabile compounds, flashes produced only an electrical artifact that was apparently caused by the 12-kV flashlamp trigger pulse; this artifact was effectively reduced by shielding and was complete in  $<1$  ms, the duration of the flash (see, for example, Fig. 1).

### *Solutions*

The bathing solution normally used had the following composition (mM): 160 NaCl, 1  $\text{MgCl}_2$ , 10  $\text{BaCl}_2$ , 10 glucose, 5 Hepes (pH 7.4). Although  $\text{Ca}^{2+}$  (10 mM) replaced  $\text{Ba}^{2+}$  in some experiments,  $\text{Ba}^{2+}$  was preferred because, with  $\text{Ba}^{2+}$  as the current carrier, the amplitude of  $I_{si}$  is larger (Kass and Tsien, 1975; Reuter and Scholz, 1977) and its rate of inactivation is greatly reduced (Noble and Yahkin, 1981; Lee and Tsien, 1983). In most cases, pipettes were filled with (mM) 140 CsCl, 10 EGTA, 10 glucose, 5 Hepes (pH 7.4), to suppress outward currents.

A stock solution of nifedipine (25 mM) was prepared by dissolving the crystals in dimethylsulfoxide (DMSO); the stock is stable in the dark for at least 3 mo. For experiments, the stock was serially diluted with the bathing solution. The DMSO concentration in the bathing solution never exceeded 0.05% in our experiments; this concentration of DMSO by itself had no measurable effects on ionic currents recorded either in the absence or in the presence of light.

### Simulations

Numerical simulations of  $\text{Ba}^{2+}$  currents recorded experimentally in the absence and presence of nifedipine, before and after flashes, were performed with the TUTSIM program (Applied i, Palo Alto, CA), which integrates linear differential equations. A more complete description of the model and the parameters used is given in the Appendix.

### RESULTS

When  $I_K$  and  $I_{\text{Na}}$  were blocked as described in the Methods,  $I_{\text{si}}$  was readily recorded from single, cultured ventricular myocytes in the presence of  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$  as the current carrier, using the whole-cell recording technique. Fig. 1A shows currents evoked, in the presence of 10 mM  $\text{Ba}^{2+}$ , by step depolarizations

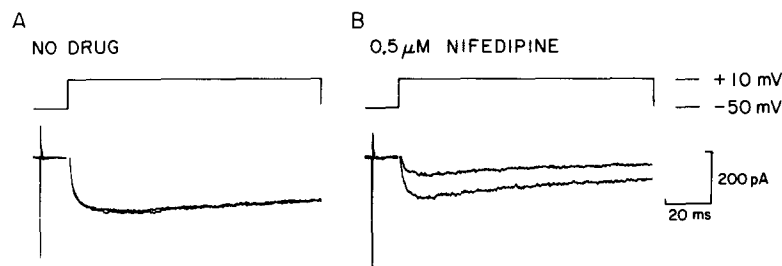


FIGURE 1. Photoremoval of nifedipine blockade of inward  $\text{Ba}^{2+}$  currents, which were elicited by stepping from a holding potential of  $-50$  to  $+10$  mV. Leakage currents have been subtracted. Each panel shows two superimposed consecutive current traces, evoked at an interval of 5 s. During the second sweep only, a flash was presented at the time indicated by the brief electrical artifact. In the absence of nifedipine (A), the flash had no effect on the current. In the presence of  $0.5 \mu\text{M}$  nifedipine (B), the current was suppressed; in this case, the flash increased the current amplitude by reversing the nifedipine blockade.

from a holding level of  $-50$  to  $+10$  mV at 5-s intervals; the  $\text{Ba}^{2+}$  currents rose to a peak with a half-time of  $\sim 2$  ms and inactivated slowly. During the second trial, a single flash was delivered; apart from the brief electrical artifact observed, the currents recorded in the two trials superimposed. Thus, in the absence of nifedipine, the flash had no effect on  $I_{\text{si}}$ . This was a consistent finding under all experimental conditions regardless of when the flash was delivered, i.e., before or during depolarizing steps.

### Suppression of $I_{\text{si}}$

Nifedipine, at concentrations of  $>10$  mM, reduced the amplitude of  $I_{\text{si}}$ . In the presence of  $0.5 \mu\text{M}$  nifedipine,  $\text{Ba}^{2+}$  currents were markedly reduced (Fig. 1B) compared with those measured in the absence of drug (Fig. 1A); this blockade was reversed by flashes. At concentrations of  $\leq 0.5 \mu\text{M}$ , a single flash, when delivered before or within the first few milliseconds of the voltage step, caused nearly complete reversal of the nifedipine-induced suppression of  $I_{\text{si}}$ . Thus, the amount of recovery from block, and hence the percentage of block produced by nifedipine, could be estimated after flashes. This method, an alternative to

measuring nifedipine suppression of  $I_{si}$  by bath application of drug followed by washout, offered the advantage that recovery was measured as the difference in  $I_{si}$  amplitudes recorded seconds apart. These measurements did not rely on drug washout, which is slow, especially at high nifedipine concentrations. In addition, these experiments were not complicated by rundown of the current (Kostyuk, 1981), which is usually encountered in whole-cell recordings of  $Ca^{2+}$  currents and which can be highly variable in magnitude and rate. The fractional inhibition of  $I_{si}$ , measured from the recovery of  $I_{si}$  amplitude after a single flash, is plotted as a function of nifedipine concentration in Fig. 2. Interpolation provides an

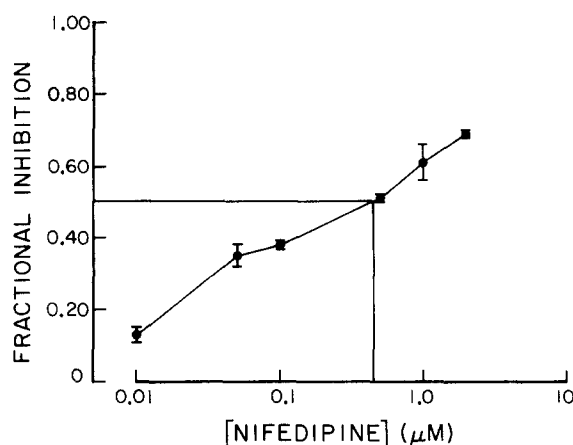


FIGURE 2. Blockade of  $Ba^{2+}$  currents by nifedipine. Currents were elicited by depolarizations to +10 mV from a holding potential of -50 mV. Fractional inhibition, measured from the increase in  $I_{si}$  amplitude produced by a single flash presented a few milliseconds before the start of the voltage step, is plotted as a function of nifedipine concentration. Interpolation provides an  $EC_{50}$  value of 450 nM for the suppression of  $Ba^{2+}$  current amplitude, which reflects closed channel block by nifedipine.

estimate of the concentration producing 50% inhibition ( $EC_{50}$ ) of  $I_{si}$  amplitude of  $450 \pm 60$  nM, a value similar to those reported for nifedipine in other preparations and for other dihydropyridine derivatives (Lee and Tsien, 1983; Kass, 1983; Morad et al., 1983; Sanguinetti and Kass, 1984b; Nerbonne et al., 1985). With  $Ca^{2+}$  (10 mM) as the current carrier, 0.5  $\mu$ M nifedipine also caused an ~50% reduction of  $I_{si}$  amplitude, although extensive concentration-response studies have not been completed. Preirradiated solutions of nifedipine, at concentrations of up to 10  $\mu$ M, had no measurable effects on  $I_{si}$  either in the absence or in the presence of flashes.

#### *Block Is Independent of Test Potential*

Currents were recorded during successive depolarizations to various levels from a holding potential of -50 mV, with either  $Ba^{2+}$  (10 mM) or  $Ca^{2+}$  (10 mM) in the bath, both in the absence and presence of nifedipine.  $I_{si}$  was routinely

observed on depolarizations to potentials more positive than  $-20$  mV; current amplitude reached a maximum at approximately  $+10$  mV. Peak amplitudes of  $I_{si}$  are plotted in Fig. 3 as a function of test potential; the data obtained from several cells were combined and averaged by first normalizing the peak currents measured at each potential to that observed during a step to  $+10$  mV. It is clear that the peak amplitude of  $I_{si}$ , for both  $Ba^{2+}$  and  $Ca^{2+}$  currents, was reduced equally at all potentials by the addition of  $0.5 \mu M$  nifedipine. Similar results were obtained at all nifedipine concentrations tested in the range  $10$  nM to  $2 \mu M$  (data not shown) and it is clear that the reduction of  $I_{si}$  amplitude caused by nifedipine is independent of test potential.

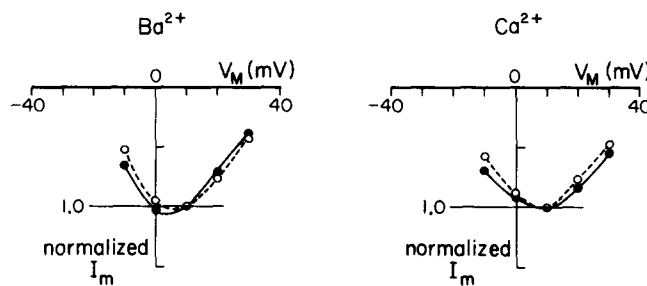


FIGURE 3. Current vs. voltage relationships for  $I_{si}$ . Currents were elicited by voltage steps from a holding potential of  $-50$  mV to various test potentials in the presence of either  $Ba^{2+}$  or  $Ca^{2+}$  ( $10$  mM). The peak amplitude of currents measured in the absence (●) and in the presence (○) of  $0.5 \mu M$  nifedipine was normalized to the amplitude measured at a test potential of  $+10$  mV. The mean values from several cells have been plotted. The current vs. voltage relationship for  $I_{si}$  was unchanged by nifedipine whether  $Ba^{2+}$  or  $Ca^{2+}$  carried the current.

#### *Time Course of Activation*

As mentioned previously, when the current through the slow channels is carried by  $Ba^{2+}$ , the amplitude of  $I_{si}$  is larger (Kass and Tsien, 1975; Reuter and Scholz, 1977) and the rate of inactivation is greatly reduced (Noble and Yahkin, 1981; Lee and Tsien, 1983). Beginning  $\sim 2$  ms after the start of the voltage step, the rising phase of  $I_{si}$  was fit with a single exponential (Bean et al., 1984) and the  $I_{si}$  activation rate constants were estimated. The rate constants for current activation were measured at various test potentials in the presence and absence of  $0.5 \mu M$  nifedipine (Fig. 4). As shown here, the rate constants for  $I_{si}$  activation varied with test potential, but were unaffected by nifedipine at all potentials examined. These results are similar to earlier findings in single dialyzed guinea pig ventricular cells, where  $I_{si}$  activation kinetics were unaffected by nitrendipine and other organic (nondihydropyridine) calcium antagonists (Lee and Tsien, 1983). Similar results were obtained here when  $Ca^{2+}$  was the current carrier, although in this case the rate constants could not be measured so accurately.

#### *Speed of Reactivation after Flashes*

How rapidly does the calcium conductance reactivate after flash-induced removal of nifedipine? The results of experiments designed to examine this question are

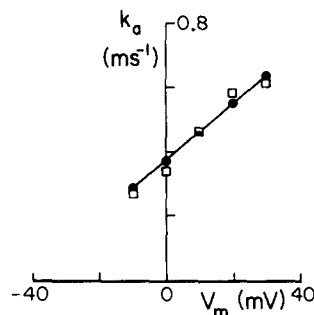


FIGURE 4. Rate constants for activation of  $\text{Ba}^{2+}$  currents ( $k_a$ ), estimated from a single-exponential fit to the rising phase of  $I_{si}$  beginning 2 ms after the voltage step, are plotted as a function of the test potential ( $V_m$ ) in the absence (●) and presence (□) of  $0.5 \mu\text{M}$  nifedipine. The rate constants increased with depolarization, but were unaffected by nifedipine at all potentials tested.

shown in Fig. 5. In the presence of  $0.5 \mu\text{M}$  nifedipine,  $I_{si}$ , measured in  $\text{Ba}^{2+}$  (A) and  $\text{Ca}^{2+}$  (B), was evoked by depolarizations to  $+10 \text{ mV}$  from  $-50 \text{ mV}$  at intervals of 5 s. During the second trial, a single flash was presented near the peak current and, as a result,  $I_{si}$  amplitude increased. This increase occurred at

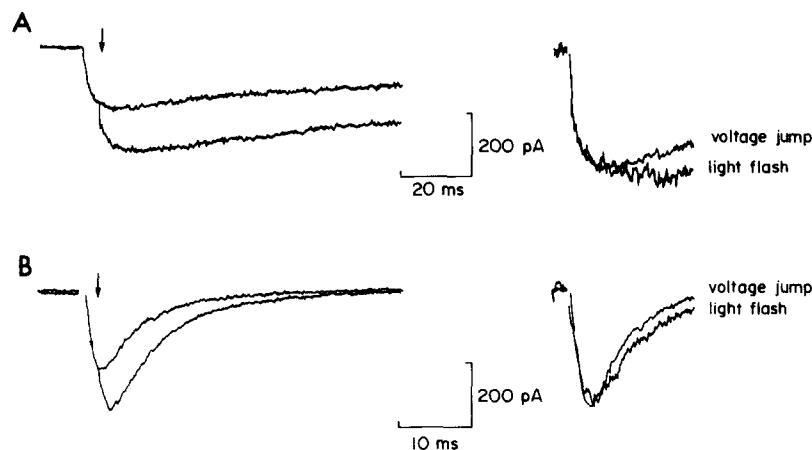


FIGURE 5. Comparison of voltage-jump and light-flash kinetics. The left-hand panels show  $\text{Ba}^{2+}$  (A) and  $\text{Ca}^{2+}$  (B) currents, elicited by voltage steps from  $-50$  to  $+10 \text{ mV}$ , in the presence of  $0.5 \mu\text{M}$  nifedipine. In both cases, two consecutive currents, evoked 5 s apart, are superimposed. During the second trial only, a flash was delivered at the time indicated by the arrow, which resulted in an increased inward current. Subtracting the current recorded in the first trial from that in the second trial gave the flash-induced current. The  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  currents measured before and after the flash were scaled, shifted in time, and superimposed in the panels on the right. The rising phase of the flash-induced current parallels the rising phase of the voltage-jump-induced current with both  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  currents, which reveals that, after a flash, the currents increase with the normal rate of activation. Similar results were obtained at all test potentials.



a rate equal to the normal rate of current activation following the voltage step. Similar findings were obtained in both  $\text{Ca}^{2+}$ - and  $\text{Ba}^{2+}$ -containing solutions. Scaling and superimposing the currents before and after a flash clearly demonstrates this result (Fig. 5, right panels).

The rate constants for the reactivation of  $I_{\text{si}}$  after flashes were measured and compared with the normal rate constants for current activation, measured after a voltage step, as a function of the time during the trial that the flash was delivered (see Table I). The rate constants for the activation of  $\text{Ba}^{2+}$  currents were estimated as described earlier. Similar calculations and comparisons were more complicated with  $\text{Ca}^{2+}$  because of the speed of  $\text{Ca}^{2+}$  current inactivation, which is apparently rapid enough to overlap with activation. Nevertheless, rough estimates of the rate constants were made after subtraction of the inactivating phase. The results confirmed that when a flash was presented before, at, or a

TABLE I  
*Comparison of Voltage-Jump and Light-Flash Kinetics*

Current carrier	Rate constant of relaxation		Flash delay after voltage jump
	Voltage jump	Light flash	
	$\text{ms}^{-1}$	$\text{ms}^{-1}$	$\text{ms}$
$\text{Ba}^{2+}$	$0.42 \pm 0.03$ (16)	—	—
		$0.46 \pm 0.03$ (17)	0
		$0.35 \pm 0.04$ (15)	5–10
		$0.32 \pm 0.09$ (10)	15–25
$\text{Ca}^{2+}$	$1.9 \pm 0.2$ (3)	—	—
		2.0 (2)	0
		$1.5 \pm 0.1$ (5)	1–4
		$1.9 \pm 0.2$ (3)	5–10

Mean  $\pm$  SEM. The number of observations is in parentheses.

few milliseconds after the peak of  $I_{\text{si}}$ , the current increased at the same rate as the normal rate of current activation for both permeant ions.

Although it could be argued that a rapid component in the recovery of  $I_{\text{si}}$  might not have been detected when the current carrier was  $\text{Ca}^{2+}$ , the relaxation of  $\text{Ba}^{2+}$  currents was slow enough that a rapid component would have been resolved if significant. Photoconversion of nifedipine is complete in  $<100 \mu\text{s}$  (Morad et al., 1983) and therefore within the 1-ms duration of the flash. Photoinduced removal of nifedipine from open channels would therefore be expected to reveal an “instantaneous” ( $\sim 1 \text{ ms}$ ) increase in  $I_{\text{si}}$  amplitude; this effect might be expected to be most pronounced at or near the peak of  $I_{\text{si}}$  when the maximal fraction of channels is activated. The observation that the rates of reactivation of  $I_{\text{si}}$  paralleled the normal rates of current activation suggest that, rather than acting to block open  $\text{Ca}^{2+}$  channels, the principal action of nifedipine is to bind to and block closed  $\text{Ca}^{2+}$  channels.

#### *Acceleration of $I_{\text{si}}$ Decay*

It has been suggested (Lee and Tsien, 1983; Sanguinetti and Kass, 1984b) that at least part of the antagonist activity of the dihydropyridines can be ascribed to

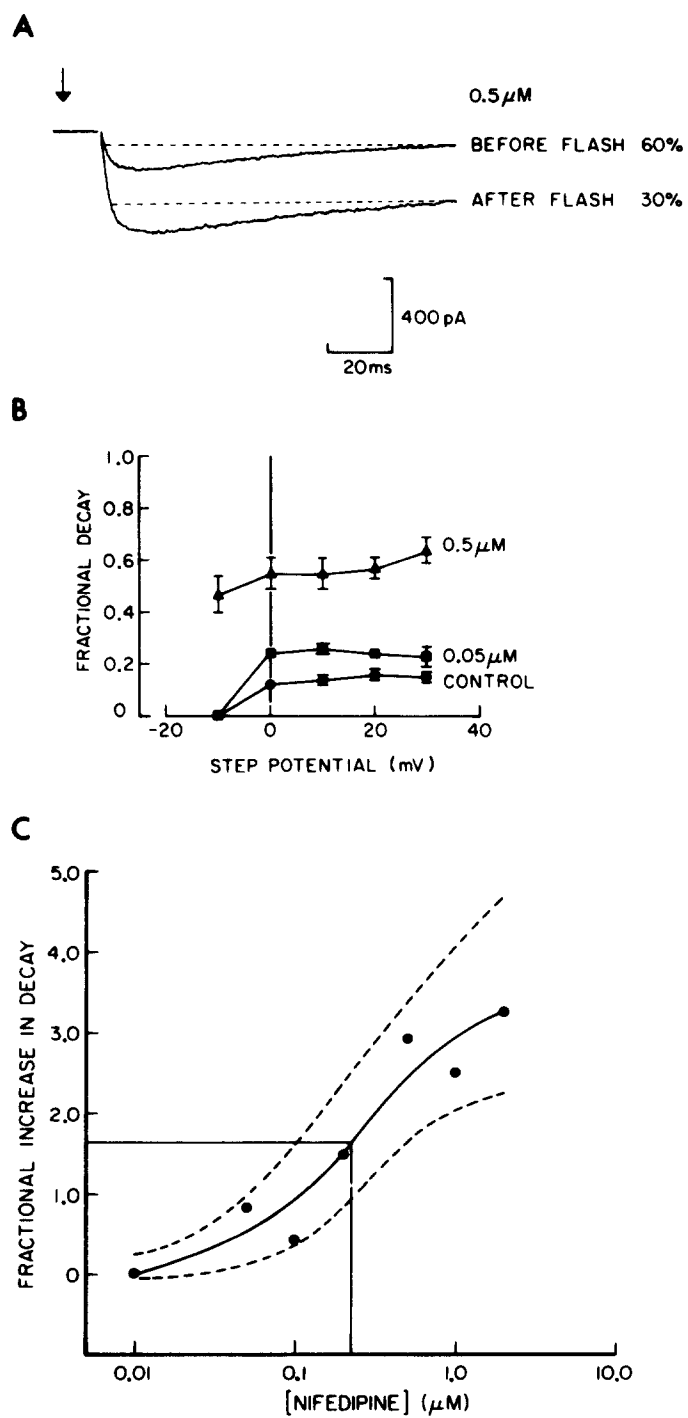


FIGURE 6.

blockade of open channels. Although open channel block was not revealed when flashes were presented before or at the peak of  $I_{si}$ , it may nevertheless have been present. Although the rates of current activation were unaffected, nifedipine accelerated the decay of  $Ba^{2+}$  currents (Fig. 6A). In the presence of  $0.5 \mu M$  nifedipine, currents declined by  $\sim 60\%$  during the 115-ms depolarization; after a flash, this was reduced to  $\sim 30\%$ . Fig. 6B compares the effects of nifedipine on the falling phase of  $Ba^{2+}$  currents during steps to various potentials. In the absence of the drug, currents decayed by  $<15\%$  at all potentials tested, although this value tended to increase slightly with depolarization. Nifedipine, in a concentration-dependent fashion, accelerated the decline and did so similarly at all potentials. This effect, revealed only after channels were opened, is reminiscent of results reported previously for suppression of  $I_{si}$  by dihydropyridines and other calcium antagonists (Lee and Tsien, 1983; Sanguinetti and Kass, 1984b). Although in these previous studies this effect was proposed to reflect open channel blockade, it could arise from accelerated inactivation of open channels. Results presented later will provide evidence for the former mechanism, so for the present it will be assumed that enhancement of  $Ba^{2+}$  current decay arises from blockade of open channels.

In Fig. 6C, the fractional increase in current decay, measured at 115 ms, is plotted as a function of nifedipine concentration; values obtained at all test potentials are included. Although the data are not complete enough to determine accurately the  $EC_{50}$  for this effect, a minimum value of  $\sim 200$  nM is extrapolated by assuming that the maximum effect occurred at the highest test concentration of  $2 \mu M$ . This value is comparable to the  $EC_{50}$  calculated for closed channel block (see Fig. 2). From the acceleration of  $I_{si}$  decay by nifedipine, it is possible to estimate the rate of open channel blockade. In the absence of drug,  $\sim 20\%$  of  $Ba^{2+}$  currents showed no measurable decay during 500-ms depolarizations to  $+10$  mV. Nifedipine enhanced the decay of these currents; the decline was approximately exponential after the peak, as illustrated in Fig. 7A. Time con-

FIGURE 6. (*opposite*) Effect of nifedipine on the decay of  $Ba^{2+}$  currents. A shows  $Ba^{2+}$  currents elicited at 5-s intervals by voltage steps to  $+10$  mV from a holding potential of  $-50$  mV, in the presence of  $0.5 \mu M$  nifedipine before and after a flash. Before the flash, the current decayed to  $\sim 60\%$  of its peak value during a 115-ms depolarization. After the flash, the current decayed to 30% of the peak over the same period. In B, the fractional decay of the  $Ba^{2+}$  current, measured at the end of a 115-ms depolarization to various test potentials from a holding potential of  $-50$  mV, is plotted as a function of the test potential in the absence ( $\bullet$ ) and in the presence of 50 ( $\blacksquare$ ) and 500 ( $\blacktriangle$ ) nM nifedipine. Nifedipine accelerated current decay in a concentration-dependent manner, although the magnitude of this effect was similar at all test potentials. C shows a plot of the fractional increase in  $Ba^{2+}$  current decay, defined as  $(D_N - D_C)/D_C$ , where  $D_N$  and  $D_C$  are the percent decay of the current in the presence and absence of nifedipine, respectively, during a 115-ms depolarizing step, vs. nifedipine concentration. The broken lines represent limits of error. Assuming a maximal increase in current decay at  $2 \mu M$  nifedipine, a minimum estimate of 200 nM for the  $EC_{50}$  for this effect is calculated.

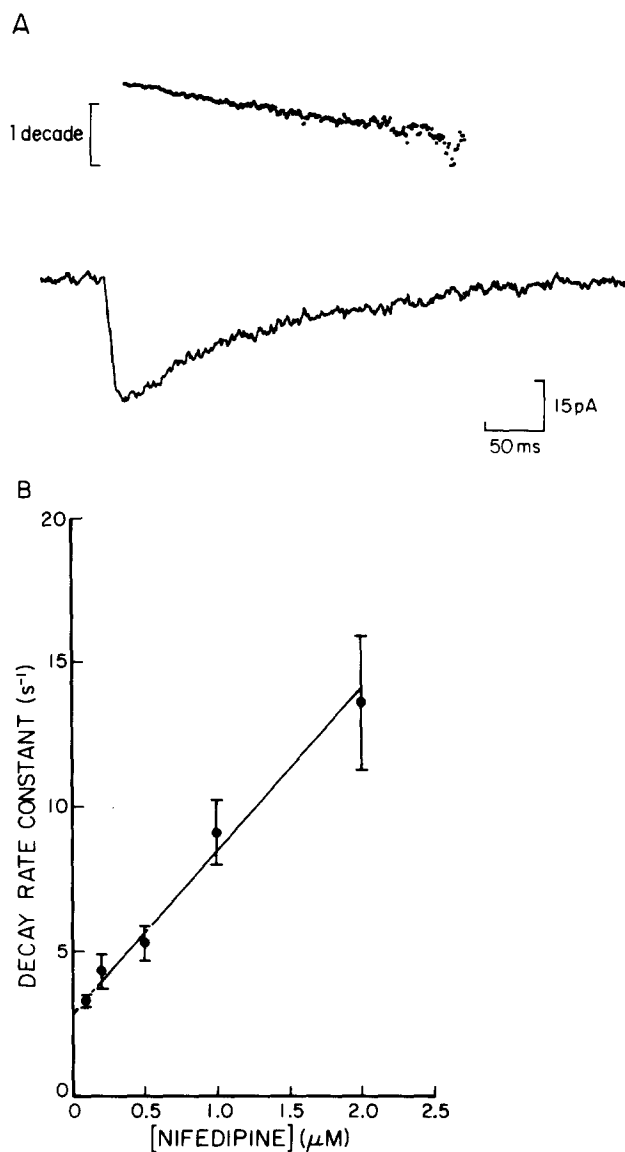
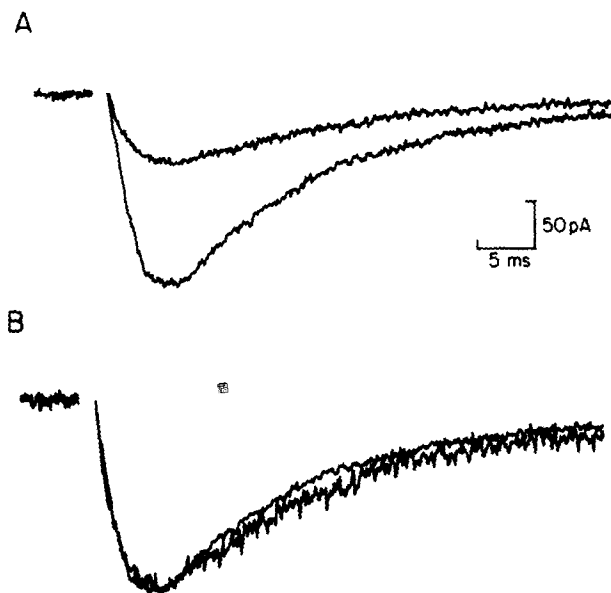


FIGURE 7. Rate constants calculated for the decay phase of  $\text{Ba}^{2+}$  currents in the presence of nifedipine. In *A*, a  $\text{Ba}^{2+}$  current recorded in the presence of  $1 \mu\text{M}$  nifedipine, in response to a step depolarization to  $+10 \text{ mV}$  from a holding potential of  $-50 \text{ mV}$ , is shown. The recorded current is displayed normally in the lower panel, while a semilogarithmic plot of the decay phase is shown above. The decay is approximated by a single exponential. In *B*, the decay rate constants (calculated as the inverses of the time constants measured from the semilogarithmic plots in *A*) are plotted as a function of nifedipine concentration. The relationship is linear over this range of concentrations. The slope, calculated by a least-squares fit to the data, provided an estimate of the rate constant for open channel blockade of  $5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ . The y-intercept provides an estimate of  $3 \text{ s}^{-1}$  for dissociation of nifedipine.

stants for current decay in the presence of various concentrations of nifedipine were therefore determined from only those currents that were fit by a single exponential. At nifedipine concentrations below  $1 \mu\text{M}$ , the current did not decay completely to zero; these decays were therefore fit to an exponential plus a constant. The time constants thus measured clearly reflected the fastest component of more complex decays, but, since the simpler decays are less likely to be contaminated by processes other than blockade, they were preferred for kinetic analysis. The decay rate constants (the inverses of the measured time constants)



**FIGURE 8.** Influence of nifedipine on the waveform of  $\text{Ca}^{2+}$  currents. Nifedipine had little effect on the decay of  $I_{\text{si}}$  recorded in the presence of  $10 \text{ mM } \text{Ca}^{2+}$ . During a 46-ms depolarizing step to  $+10 \text{ mV}$  from a holding potential of  $-50 \text{ mV}$ , the  $\text{Ca}^{2+}$  current amplitude decayed to  $\sim 90\%$  of the peak in the presence of  $0.5 \mu\text{M}$  nifedipine (A, smaller trace). After a flash, although the peak amplitude of the current was enhanced, current decay was similar (A, larger trace). This is demonstrated more clearly when the currents are scaled and superimposed (B). Similar results were obtained at all nifedipine concentrations and all test potentials examined (data not shown).

were linearly related to nifedipine concentration, as shown in the plot in Fig. 7B. The slope, calculated by a linear least-squares fit to the data, provided an estimate of the rate constant for blockade of open channels of  $5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ , while the zero-concentration intercept of  $3 \text{ s}^{-1}$  is presumably dominated by unblocking. The ratio of the two values suggests an equilibrium constant of  $560 \text{ nM}$ , although the exact interpretation depends on the molecular model for binding.

Fig. 8 shows the results of similar studies with  $\text{Ca}^{2+}$  as the current carrier. Calcium currents, evoked by depolarizations to  $+10 \text{ mV}$  from a holding potential

of  $-50$  mV, declined by 90% within  $\sim 46$  ms. These currents decayed at a substantially greater rate than the extra decay induced by nifedipine when  $\text{Ba}^{2+}$  carried the current. Nifedipine would therefore not be expected to modify significantly the waveform of the  $\text{Ca}^{2+}$  current. Indeed, in the presence of  $0.5$   $\mu\text{M}$  nifedipine (or at higher concentrations; data not shown), scaled episodes nearly superimpose before and after photoremoval of nifedipine.  $\text{Ca}^{2+}$ -mediated inactivation therefore dominated the decay of  $I_{\text{si}}$ , even in the presence of nifedipine, when  $\text{Ca}^{2+}$  carried the current. Thus, nifedipine had a much greater effect on the falling phase of  $I_{\text{si}}$  when the current was carried by  $\text{Ba}^{2+}$  rather than  $\text{Ca}^{2+}$ .

#### *Time Dependence of Recovery after a Flash*

The results already presented demonstrate that recovery from nifedipine-induced suppression of  $I_{\text{si}}$  could be effected when a flash was delivered before or at the peak of the current. Fig. 9 shows the recovery observed when the flash was presented later in a trial, i.e., during the decaying phase of the current. As demonstrated previously, when the flash was presented either before or at the peak of  $I_{\text{si}}$ , current amplitude was completely recovered (Fig. 9, A and B). In contrast, when the flash was presented at times later than the peak of  $I_{\text{si}}$ , only incomplete reversal of nifedipine block was observed in the episode in which the flash was presented (Fig. 9, C and D). When the flash was presented early, the third episode, recorded 5 s after the flash episode, showed reblocking as nonirradiated nifedipine diffused to the cell. With late flashes, however, the third episode showed a further increase in  $I_{\text{si}}$  amplitude. The fractional recovery observed immediately after a flash was therefore clearly dependent on when, during the depolarizing step, the flash was delivered: the later the flash was presented, the less effective it was at reversing blockade. This decline in fractional recovery occurred with a half-time of  $\sim 30$ – $40$  ms and did not appear to vary much with nifedipine concentration, although extensive studies have not been completed. These results can be explained if, after photoremoval of nifedipine, the affected channels also required membrane repolarization in order to conduct. This requirement for repolarization after a long depolarization suggests that the channels entered an inactivated state. Repolarization returned these inactivated channels to the resting, closed state and they were therefore able to open (having been unblocked) during the subsequent voltage step.

Interestingly, when flashes were presented late during depolarizing steps, there appeared to be an “instantaneous” component to the increase in  $I_{\text{si}}$  amplitude. This effect, expected if the flash reversed nifedipine blockade of open channels, supports the suggestion that such a mechanism contributes to the acceleration of  $\text{Ba}^{2+}$  current decay by nifedipine. However, this apparent rapid component of recovery was small and could not easily be quantified; its presence is not entirely convincing. The contribution of open channel blockade to nifedipine action was therefore better estimated from the current decay measurements.

It has been proposed (Bean, 1984; Sanguinetti and Kass, 1984b) that blockade by dihydropyridines results from binding to inactivated  $\text{Ca}^{2+}$  channels as it is enhanced by membrane depolarization. In order to study the influence of holding

potential on the magnitude of nifedipine blockade, we measured the effect of a prior depolarization (250 ms) to various potentials on the current recorded at +10 mV (see Fig. 10A). When prepulses were longer than 250 ms and to potentials more positive than -30 mV, extensive and often irreversible (even

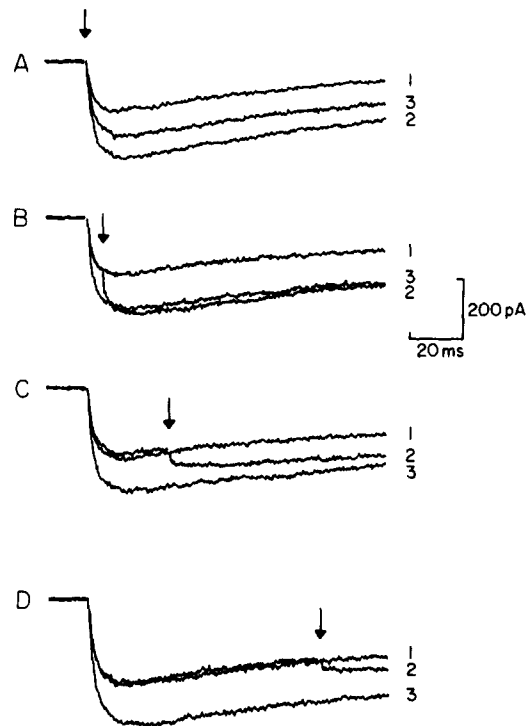
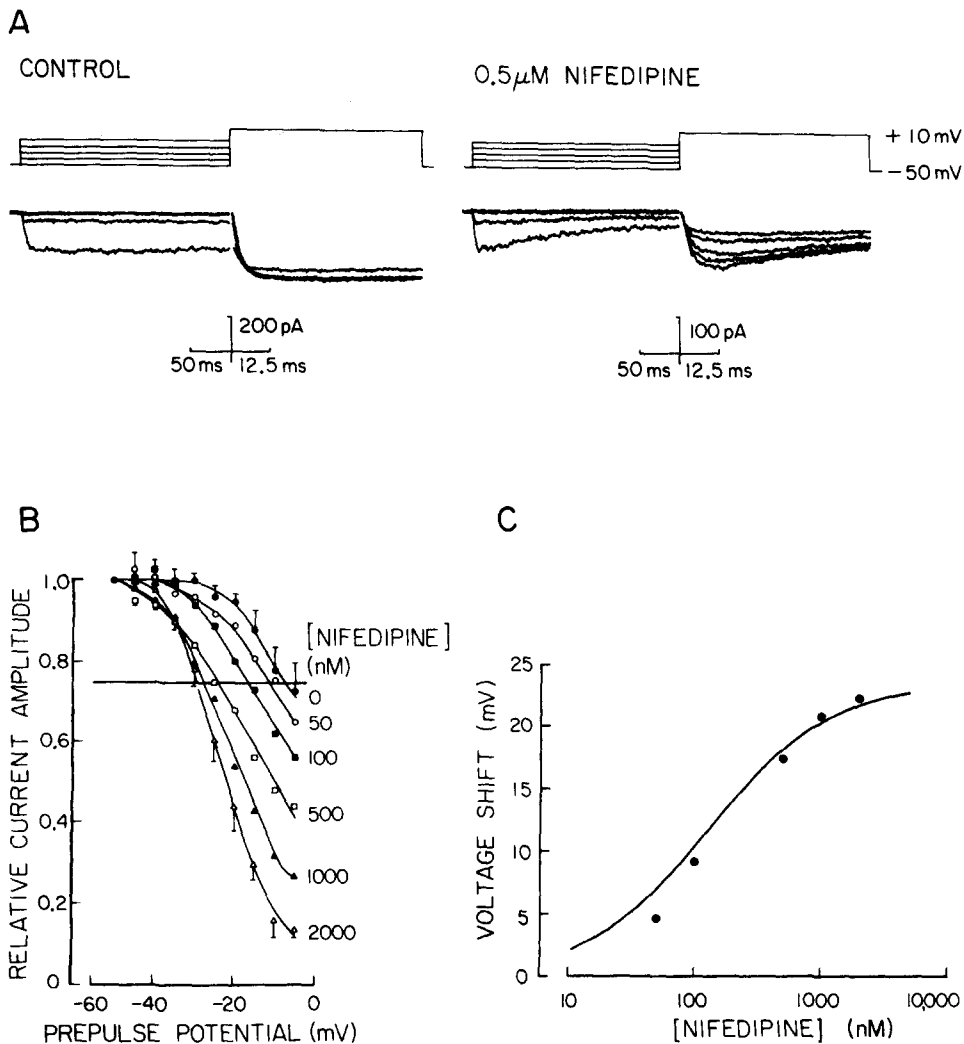


FIGURE 9. Recovery depends on the timing of the flash. In each case, three  $\text{Ba}^{2+}$  currents (labeled 1, 2, and 3), evoked 5 s apart by voltage steps from -50 to +10 mV in the presence of 0.5  $\mu\text{M}$  nifedipine, are superimposed. In each panel, a single flash was delivered during the second trial only, at the times indicated by the arrows. When the flash was delivered before or at the peak of  $I_{\text{si}}$  (A and B), the current increased, and, in the third trial, reblocking was evident as nonirradiated nifedipine diffused to the cell. When the flash was presented after the peak current (C and D), it was less effective at removing nifedipine blockade; the third trial showed a further increase in  $I_{\text{si}}$  amplitude. In some experiments, when the flash was presented late during the voltage step, there also appeared to be a step-like component to the flash-induced current (D), a finding that suggests that a small component of the recovery may be attributed to reversal of open channel block (see Discussion).

after the membrane had been repolarized for several seconds) inactivation of  $I_{\text{si}}$  resulted. This effect appeared to be an accelerated form of current "rundown" (Kostyuk, 1981), which suggests that  $\text{Ca}^{2+}$  current rundown in these dialyzed cells may be voltage-sensitive. Although the prepulse may have been too brief to produce "steady state" inactivation, it was sufficiently long to cause some inactivation at potentials more positive than -25 mV. Nifedipine caused a concentra-



**FIGURE 10.** Effect of a long prepulse on  $\text{Ba}^{2+}$  current amplitude. The experimental protocol is illustrated in **A**. From a holding potential of  $-50$  mV, 250-ms prepulses to various potentials were applied before depolarization to the test potential of  $+10$  mV. In the absence of nifedipine, little inactivation of the  $\text{Ba}^{2+}$  current was observed during prepulses to  $-45$  to  $-20$  mV, as evidenced by the superposition of the currents elicited by the test depolarization. In the presence of  $0.5 \mu\text{M}$  nifedipine, however, a prepulse to  $-30$  mV was sufficient to cause substantial inactivation. In **B**, the peak amplitudes of  $I_{\text{si}}$ , elicited from various prepulse potentials, have been normalized to the amplitude measured when  $I_{\text{si}}$  was evoked directly from  $-50$  mV, and are plotted as a function of prepulse potential. Results obtained in the presence of various nifedipine concentrations are shown; the horizontal line is drawn at 25% inactivation. The voltage shift of the curves in **B**, at 25% inactivation, is plotted as a function of nifedipine concentration in **C**. The solid curve represents the best fit of Eq. 2 to the data and provides an estimate of  $K_1 = 39$  nM.



tion-dependent shift in inactivation to more negative potentials (Fig. 10B), which supports the hypothesis that nifedipine binds to and stabilizes channels in the inactivated state. The curves describing the availability of  $\text{Ca}^{2+}$  channels at various potentials, in the absence and presence of nifedipine (Fig. 10B), were each fit according to the conventional expression (Hodgkin and Huxley, 1952):

$$I = 1 / \{1 + \exp[(V - V_h)/k]\}, \quad (1)$$

where  $I$  is the relative amplitude of the test current,  $V$  is the prepulse potential,  $V_h$  the midpoint voltage, and  $k$  is the slope factor. Increasing the nifedipine concentration shifted  $V_h$  in the hyperpolarizing direction with no consistent change in the steepness of the curves, the mean value of  $k$  being  $9.4 \pm 0.6$  mV. This is higher than the value of 4.1 mV estimated previously from steady state availability curves (Sanguinetti and Kass, 1984b) and probably reflects the much shorter prepulse used in the present experiments. The shift in the midpoint of the steady state availability curve ( $\Delta V_h$ ) is related to the nifedipine concentration ( $N$ ) and the dissociation constants for binding to resting ( $K_R$ ) and inactivated ( $K_I$ ) channels as follows (Bean et al., 1983):

$$\Delta V_h = k \ln[(1 + N/K_R)/(1 + N/K_I)]. \quad (2)$$

The magnitude of the voltage shift, measured at 25% inactivation, is plotted in Fig. 10C as a function of the nifedipine concentration and is compared with a solid curve derived from Eq. 2 with  $k = 9.4$  mV and  $K_R = 450$  nM. The best fit to the data was obtained with  $K_I = 39$  nM, which suggests that nifedipine binds more tightly to calcium channels when they are inactivated than when they are in either the resting or open states. The fit is reasonably good considering that the measured inactivation is unlikely to reflect the true "steady state" value (because the prepulse lasted only 250 ms).

#### DISCUSSION

The results presented here demonstrate that flash-induced removal of the photolabile  $\text{Ca}^{2+}$  antagonist nifedipine from its binding site(s) can be an effective means of studying the nature of these sites. This type of experiment can thus reveal mechanistic details of drug action that are not readily provided by more conventional experimental approaches. The main conclusion of this study is that, qualitatively, nifedipine acts in a manner similar to other organic calcium antagonists such as verapamil, D600, and diltiazem, i.e., it exerts its effect(s) by interacting with  $\text{Ca}^{2+}$  channels in three kinetic states: closed, open, and inactivated. We suggest, therefore, that the actions of nifedipine, and presumably other dihydropyridine antagonists, are well described by a "modulated receptor" model (Hille, 1977; Hondeghem and Katzung, 1977), as illustrated in Fig. 11. In this scheme, three normal kinetic states of the  $\text{Ca}^{2+}$  channel are shown: the closed (C) or resting, the open (O) or activated, and the inactivated (I) states. In addition, the corresponding nifedipine-bound states—closed (CN), open (ON), and inactivated (IN)—are included. Although it is clear that more kinetic states than this certainly do exist (Fenwick et al., 1982; Bean et al., 1983; Cachelin et al., 1983; Reuter et al., 1982, 1983; Hess et al., 1984), the model has been

depicted with only three states for the sake of simplicity. As will become evident, a more complete model of the kinetic states of the  $\text{Ca}^{2+}$  channel is unnecessary for the conclusions drawn here.

Photoconversion of nifedipine, when effected before the peak of  $I_{\text{si}}$ , increased the inward current; this increase had the same rate constant as the normal, voltage-dependent rate of  $I_{\text{si}}$  activation. These findings imply that when flashes were delivered early during depolarizing steps, nifedipine was removed mainly

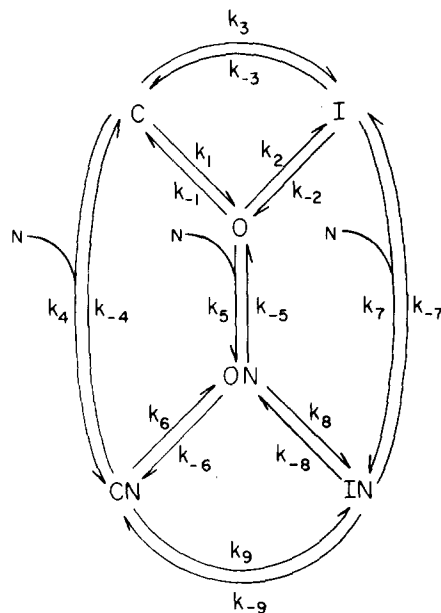


FIGURE 11. Modulated receptor model for nifedipine blockade of  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  currents. Interconversions between normal states of the  $\text{Ca}^{2+}$  channel, C (closed), O (open), and I (inactivated), and the corresponding nifedipine-blocked states, CN, ON, and IN, are shown. Although more states of the  $\text{Ca}^{2+}$  channel exist, these need not be considered in the present model. As previously suggested (Hille, 1977), it is assumed that all interconversions may in principle be voltage-dependent and that the rates and equilibria for the normal and the nifedipine-blocked states need not be the same (see Discussion and Appendix).

from closed, resting channels; the flash thus resulted in a net CN-to-C conversion, allowing the unblocked channels to open at the normal rate. The  $\text{EC}_{50}$  of  $450 \pm 60$  nM, estimated for nifedipine suppression of  $I_{\text{si}}$  amplitude (using the flash-removal paradigm described; see Fig. 2) therefore reflects the binding affinity for the closed channel. Nifedipine had no measurable effect on the kinetics of activation of  $I_{\text{si}}$ , which is consistent with the notion that the drug binds to closed channels and prevents them from opening in response to depolarization rather than altering the rate constants governing normal channel openings and/or closings.

When  $\text{Ba}^{2+}$  was the current carrier through  $\text{Ca}^{2+}$  channels, nifedipine, in addition to reducing the amplitude of  $I_{\text{si}}$ , accelerated current decay. This obser-

vation is explained if nifedipine binds to open  $\text{Ca}^{2+}$  channels (i.e.,  $\text{O} \rightleftharpoons \text{ON}$ ), thereby inhibiting current flow. Alternatively, this effect might be attributed to nifedipine-induced acceleration of the normal rate of channel inactivation. As discussed in the Appendix, the current waveform and most of the effects of flashes could be modeled with either a direct acceleration of inactivation or with open channel blockade by nifedipine. The presence of open channel block is supported by the observation that, when flashes were presented well after the peak of  $I_{\text{si}}$ , there appeared to be a small rapid component to the recovery of current amplitude. However, since this "instantaneous" current increase, which would only be expected if photolysis removed nifedipine from open channels, was small, it could not be easily quantified and was not always convincing. Thus, although the enhanced decay of  $\text{Ba}^{2+}$  currents in the presence of nifedipine is most simply explained by open channel blockade, the absence of a large instantaneous component in the flash-induced current suggests that this mechanism is unlikely to contribute significantly to the overall effects of nifedipine. As discussed in the Appendix, accelerated inactivation, which has also been proposed to explain some of the effects of nitrendipine on single  $\text{Ca}^{2+}$  channel currents (Hess et al., 1984), is a possible explanation for this effect. Open channel blockade was not revealed in the presence of  $\text{Ca}^{2+}$  as the current carrier, apparently because of the already rapid rate of  $I_{\text{si}}$  decay, as discussed before.

Presenting flashes during the decaying phase of  $\text{Ba}^{2+}$  currents revealed a third mechanism of nifedipine blockade. Flashes became increasingly less effective at reversing blockade when delivered later during depolarizing steps. This can be explained if, at long times, most of the nifedipine is bound to inactivated channels. Thus, although the flash would still remove nifedipine from the channels (IN to I), they would be unable to conduct until they underwent a further transition to the open (I to O) or closed (I to C) state (Fig. 11). This action of nifedipine was only apparent several tens of milliseconds after the peak of  $I_{\text{si}}$ , a finding that is consistent with the slow formation of blocked-inactivated channels. An alternative explanation for the incomplete recovery observed after flashes presented late during a step is that the photoproduct of nifedipine dissociates more slowly from the binding site at positive potentials. However, it seems unlikely that the binding characteristics would adjust so slowly to changes in membrane potential. The postulate that inactivated channels are blocked by nifedipine is also supported by the finding that the efficacy of nifedipine is enhanced at depolarized holding potentials.

The dissociation constant of 39 nM, which was estimated for binding to inactivated channels from the concentration dependence of the voltage shift in inactivation (Fig. 10), is lower than the concentrations required to produce 50% blockade of closed or open channels. This agrees with recent studies on other dihydropyridines (Bean, 1984; Sanguinetti and Kass, 1984b) and suggests that nifedipine binds more tightly to inactivated channels than to closed or open channels. The value of 39 nM is, however, higher than the nanomolar dissociation constants estimated in the other studies and in biochemical studies on the binding of nifedipine to isolated membranes. Bean (1984) recently suggested that this discrepancy vanishes if the membrane is held depolarized for several minutes. As noted in the Results, such measurements were vitiated in our experiments by

rapid rundown of  $I_{si}$  at depolarized voltages. It should be noted that flashes were not helpful for demonstrating block of inactivated channels when  $Ca^{2+}$  carried inward current. This can (as with open channel block) be attributed to the fact that, in  $Ca^{2+}$ , channel inactivation is already rapid in the absence of drugs. However, recent studies have shown that in the presence of dihydropyridines, inactivation of  $I_{si}$  is also shifted to hyperpolarized potentials when  $Ca^{2+}$  carries the current (Sanguinetti and Kass, 1984a), which suggests that in these conditions nifedipine may also block inactivated  $Ca^{2+}$  channels. This was not examined in the present work.

How does this blocked-inactivated channel form? Normally, when  $Ba^{2+}$  is the current carrier, open channels inactivate very slowly (O to I) and therefore very few channels inactivate during a voltage step of several hundred milliseconds. Unless nifedipine alters the rate of O to I, it is unlikely that many inactivated-blocked channels would form via the  $I \rightleftharpoons IN$  pathway. The results are therefore more readily explained if nifedipine-bound channels, either in the closed (CN) or the open (ON) state, directly or indirectly inactivate, i.e.,  $CN \rightleftharpoons IN$ ,  $ON \rightleftharpoons IN$ , or  $CN \rightleftharpoons ON \rightleftharpoons IN$ . Since the recovery produced by flashes presented before the peak of  $I_{si}$  was due mainly to the unblocking of closed channels, the inefficiency at effecting recovery later during a voltage step suggests that a large fraction of blocked-closed channels inactivate ( $CN \rightleftharpoons IN$  or  $CN \rightleftharpoons ON \rightleftharpoons IN$ ). On the other hand, assuming that the acceleration of  $Ba^{2+}$  current decay arises entirely from blockade of open channels, then if only closed-blocked channels inactivate, photoremoval of nifedipine from open channels (O to ON) would be observed as an instantaneous increase in current to a level equal to the peak amplitude of the nifedipine-blocked current. However, the current was only recovered to ~25% of the peak amplitude of the current suppressed by nifedipine ( $0.5 \mu M$ ) when a flash was delivered  $\geq 50$  ms after the start of the voltage step. This suggests that at least some nifedipine-bound open channels inactivate ( $ON \rightleftharpoons IN$ ). The modulated receptor model predicts the observed rate of formation of inactivated channels during a voltage step best if closed channels are allowed to inactivate both directly ( $C \rightleftharpoons CN$ ) and indirectly ( $CN \rightleftharpoons ON \rightleftharpoons IN$ ) (see Appendix).

It appears, therefore, that in the presence of nifedipine, closed channels can inactivate when the membrane is depolarized, without first opening. However, it is not apparent whether or not this pathway normally occurs in the absence of drug ( $C \rightleftharpoons I$ ), although this is one explanation for the kinetic behavior of single  $Ca^{2+}$  channels observed in other preparations (Lux and Brown, 1984). The present experiments were unable to evaluate directly the importance of the  $C \rightleftharpoons I$  and  $CN \rightleftharpoons IN$  pathways, but it should be possible to evaluate the net contribution of inactivation of closed channels (Lux and Brown, 1984) using patch-clamp recording techniques to evaluate single channel currents (Hamill et al., 1981; Cachelin et al., 1983; Reuter et al., 1982).

Recent observations on the behavior of single  $Ca^{2+}$  channel currents prompted the suggestion that  $Ca^{2+}$  channels have three modes of gating (Hess et al., 1984). Current records from cell-attached patches of membrane on isolated heart cells show rapid bursts of brief openings of  $Ca^{2+}$  channels in response to depolarization

(mode 1; see also Reuter et al., 1982; Cavalie et al., 1983). Occasionally, however, records are devoid of openings (mode 0) and, in rare cases, display long openings with brief closings (mode 2). Since the probability of observing no openings is greatly increased in the presence of dihydropyridine antagonists, it was proposed (Hess et al., 1984) that they act by preferentially stabilizing the channel in mode 0, thus reducing channel availability. Some dihydropyridines—nitrendipine, for example—may have mixed agonist and antagonist activity as they additionally promote mode 2 behavior. The present finding that nifedipine binds to and stabilizes channels in a closed state is consistent with the promotion of mode 0 activity. On the other hand, the observation that  $I_{si}$  amplitude recovers within a few milliseconds after photoremoval of nifedipine is not consistent with the notion that dihydropyridines merely promote a particular form of gating. Hess and co-workers (1984) showed that current records displaying mode 0 and mode 2 activity are clustered on a time scale of several seconds; they suggested that  $Ca^{2+}$  channels undergo transitions between modes of gating on this time scale. In terms of the mode model, photodestruction of nifedipine returns the gating behavior from mode 0 to mode 1; the rate of this intermode conversion, however, is several orders of magnitude faster than the rates suggested to govern such conversions. Our results therefore tend to minimize the distinction between modes of gating and a more conventional mechanism whereby nifedipine increases the number of states available to the channel (Fig. 11).

In conclusion, nifedipine suppresses currents through calcium channels of neonatal rat ventricular myocytes by interacting with closed, open, and inactivated channels. However, open channel blockade is unlikely to contribute significantly to the drug's effects, particularly when the current is carried by  $Ca^{2+}$ . At the normal resting potential of the cardiac cell, nifedipine is likely to suppress  $Ca^{2+}$  currents mainly by blocking closed channels. Nifedipine appears to bind more tightly to the channel when it is inactivated so that its blockade is enhanced at depolarized potentials. As suggested by Sanguinetti and Kass (1984b), because some vascular smooth muscle cells have depolarized resting potentials, the tight binding of dihydropyridines to inactivated channels may explain why these drugs are particularly effective vasodilators, and hence why they are particularly effective in treating such clinical disorders as angina pectoris and hypertension. Nifedipine's efficacy against angina may also be attributable in part to blockade of inactivated channels (in addition to closed channels) in the heart, which are likely to be more abundant in the damaged (depolarized), ischemic areas of the heart (Flaim and Zelis, 1981; Smith, 1983).

#### APPENDIX

The modulated receptor model, as outlined in Fig. 11, was the starting point for simulating the effects of nifedipine on  $I_{si}$  (in 10 mM  $Ba^{2+}$ ) using the TUTSIM program. Initially, the  $Ba^{2+}$  current, which would be evoked by a 100-ms step depolarization to +10 mV from a holding potential of -50 mV in the absence of nifedipine, was modeled by assuming that the  $Ca^{2+}$  channel could occupy only three states: closed (C), open (O), or inactivated (I). Although it is clear that additional states of the calcium channel exist (Reuter et al., 1982; Bean et al., 1984; Cachelin et al., 1983; Hess et al., 1984), it appears unnecessary to include them.

It was assumed that the rate constant for  $\text{Ba}^{2+}$  current activation, calculated from the rising phase of the current (Table I;  $420 \text{ s}^{-1}$ ), was uncontaminated by channel closure or inactivation, and it was used as  $k_1$  in the simulations. In addition, for simplicity, it was assumed that channels do not inactivate directly from the closed state, so that  $k_3$  and  $k_{-3}$  were zero. The remaining first-order rate constants,  $k_{-1}$ ,  $k_2$ , and  $k_{-2}$ , were those estimated to provide the most accurate representation of the current waveform. A  $\text{Ba}^{2+}$  current simulation with  $k_{-1} = 10 \text{ s}^{-1}$ ,  $k_2 = 0.5 \text{ s}^{-1}$ , and  $k_{-2} = 0.1 \text{ s}^{-1}$  is displayed in Fig. 12A. Although varying the rate constants  $k_{-1}$  and  $k_2$  dramatically influences the waveform of the current,  $k_{-2}$  has little effect (for  $k_2 \ll k_1 + k_{-1}$ ), as few channels enter the inactivated state during brief depolarizations. Comparing Fig. 12A with Fig. 1 (or Figs. 2, 5A, or 9)

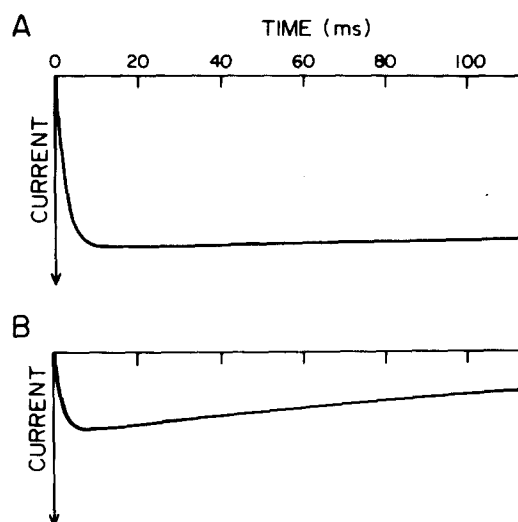


FIGURE 12. Numerical simulations of the slow inward  $\text{Ba}^{2+}$  current, using the model described in the Appendix, in the absence (A) and in the presence (B) of  $0.5 \mu\text{M}$  nifedipine. As observed experimentally, in the absence of drug, the current rises to a peak in  $\sim 12 \text{ ms}$  and decays very little during a 100-ms depolarization. The addition of nifedipine (B) to the model has little effect on the rate of current activation, although current amplitude is decreased and current decay is accelerated. The arbitrary current scale is the same in A and B.

shows that the calculations provide a fairly accurate representation of the experimentally determined  $\text{Ba}^{2+}$  currents.

When the model was extended to include the effects of  $0.5 \mu\text{M}$  nifedipine, blockade of channels in all three states, CN, ON, and IN, was assumed to be possible. The dissociation constant for binding to closed channels was set to the experimentally measured value of  $450 \text{ nM}$ . Upper limits on the rate constants for blocking and unblocking of closed channels,  $k_4$  and  $k_{-4}$ , were imposed by the experimental observation that nifedipine did not alter the rate of  $\text{Ba}^{2+}$  current activation; values of  $k_4 = 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  and  $k_{-4} = 0.9 \text{ s}^{-1}$  were determined suitable by trial and error. The rate constant for open channel block,  $k_5 = 1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ , which was found to reproduce the nifedipine-induced enhancement of current decay (see Fig. 6), is larger than that estimated from the experimental data (Fig. 8;  $5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ). This discrepancy is probably explained by errors in estimating the rate constant from only a selected subset of currents that decayed monoexponentially. The rate constant,  $k_{-5} = 3 \text{ s}^{-1}$ , for the dissociation of nifedipine from the open channel,

is the intercept on the ordinate of the plot in Fig. 8. The actual value of  $k_{-5}$  had little influence on the current waveform, provided it was small relative to the rate constant for the forward blocking reaction. It should be noted, however, that the nifedipine-blocked  $\text{Ba}^{2+}$  current could also be simulated by letting these rate constants control the inactivation step ( $\text{O} \rightleftharpoons \text{I}$ ) and by removing open channel blockade ( $\text{O} \rightleftharpoons \text{ON}$ ). Nevertheless, it was assumed initially that nifedipine did not alter the rate of channel inactivation; thus, including the  $\text{I} \rightleftharpoons \text{IN}$  interconversion, with a  $K_D$  of 39 nM as determined experimentally, did not affect these simulations, regardless of the values of the individual rate constants  $k_7$  and  $k_{-7}$ . It was also possible to calculate quite accurately the waveform of the  $\text{Ba}^{2+}$  current (Fig. 12B) in the presence of nifedipine without incorporating the  $\text{CN} \rightleftharpoons \text{ON}$ ,  $\text{ON} \rightleftharpoons \text{IN}$ , and  $\text{CN} \rightleftharpoons \text{IN}$  transitions.

The model at this stage similarly predicts the effects of photoremoval of nifedipine early in the voltage step. Flashes simulated early during the depolarization produce complete recovery of current amplitude, if all the nifedipine is destroyed, as mainly closed channels are unblocked. In the form described above, however, the model did not reproduce the experimentally observed effects of flashes delivered later during depolarizing steps. In order to simulate these results, it was necessary to populate the IN state. This can be achieved by direct ( $\text{CN} \rightleftharpoons \text{IN}$ ) and indirect ( $\text{CN} \rightleftharpoons \text{ON} \rightleftharpoons \text{IN}$ ) transitions. Although incorporation of either of these pathways would be reasonable, if acceleration of  $\text{Ba}^{2+}$  current decay arises only from blockade of open channels, then inactivation of open-blocked channels, i.e.,  $\text{ON} \rightleftharpoons \text{IN}$ , is required to model the flash effects. The following criteria were set for determining the relative contributions of the direct and indirect pathways to inactivation of nifedipine-bound closed channels. The fractional recovery produced by a flash simulated at various times during the current waveform should match the experimental observations and decay with a half-time of 30–40 ms. In addition, the current induced by flashes simulated early in the voltage step should increase at the same rate as the simulated voltage-jump-induced current. For simplicity, it was assumed that, once inactivated, the blocked channel remains in that state (i.e.,  $k_{-8}$ ,  $k_{-9} = 0$ ). The remaining rate constants governing the transitions were then varied to simulate the effects of flashes. If  $k_9 = 0$ , so that blocked-closed channels inactivate only via the  $\text{CN} \rightleftharpoons \text{ON} \rightleftharpoons \text{IN}$  pathway, then it is not possible to satisfy both criteria. If  $k_8 < k_6 + k_{-6}$ , late flashes produce too much recovery. When these three rate constants are varied to simulate the amplitude of the experimentally observed flash-induced recovery at different times, responses to early flashes have a large instantaneous component. On the other hand, if inactivation is allowed to occur only by the direct route ( $\text{CN} \rightleftharpoons \text{IN}$ ), by setting  $k_6$  and  $k_{-6}$  to zero, the value of  $k_9$  required to simulate recovery late in the pulse produces too little recovery in response to early flashes. Varying  $k_8$  had only a small effect in this instance, as only a small fraction of channels entered the blocked-open state during the pulse. Thus, these simulations suggest that nifedipine-bound channels inactivate via both routes during a depolarizing voltage step. The combination of rate constants found by trial and error to best reproduce the experimental observations (Fig. 13) was  $k_6 = 20 \text{ s}^{-1}$ ,  $k_{-6} = 2 \text{ s}^{-1}$ ,  $k_8 = 30 \text{ s}^{-1}$ , and  $k_9 = 20 \text{ s}^{-1}$ . It is interesting that currents produced by flashes simulated late in the pulse rise more rapidly (Fig. 13, B and C), which suggests that the small, rapid increase in current, suggested in the Results to be present in the experimental data, may indeed reflect recovery from open channel blockade. Other combinations of rate constants were possible, however, and they did not always result in such a large rapid component of recovery when flashes were simulated late in the episode. Neither the current waveform nor the effects of flashes were influenced by varying the equilibrium dissociation constant,  $K_7 = k_{-7}/k_7$ , for binding to inactivated channels ( $\text{I} \rightleftharpoons \text{IN}$ ) over a wide range. Thus, unfortunately, these simulations were not helpful in determining the relative affinities of nifedipine for the  $\text{Ca}^{2+}$  channel in its various states.

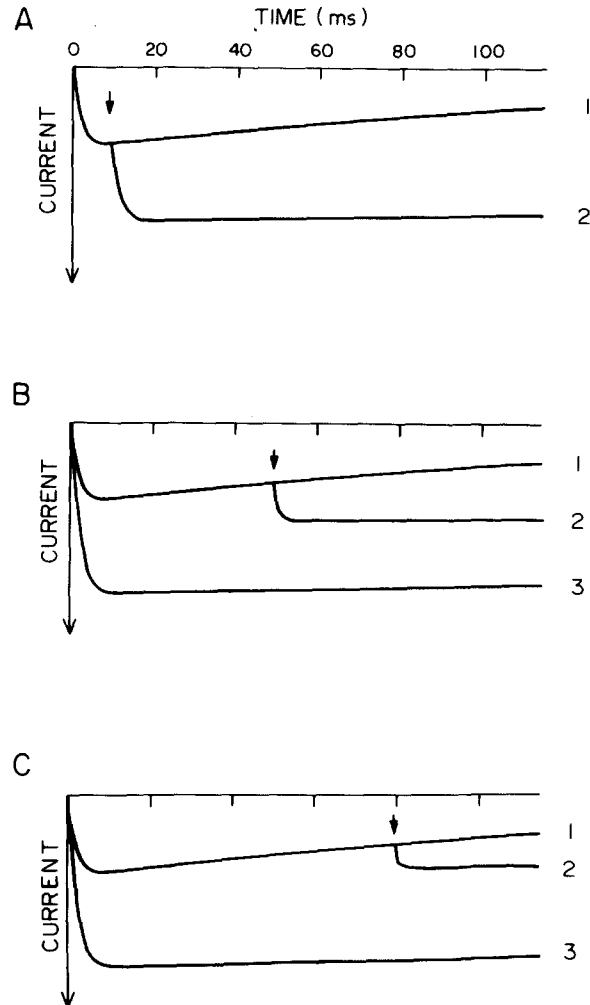


FIGURE 13. Simulations of the influence of flashes on the recovery from nifedipine blockade of the  $\text{Ba}^{2+}$  current. Rate constants are as described in the Appendix for the modulated receptor model. The lowest-amplitude trace in each case (labeled 1) represents the nifedipine-blocked current. Flashes (represented by arrows) were simulated during the traces labeled 2. The post-flash episodes (labeled 3) were simulated by assuming that the flash removed all the nifedipine from the channels and that, between traces 2 and 3, any inactivated channels were returned to the resting, closed state. When a flash is presented at the peak of the current (*A*), closed channels are unblocked. These channels open with the normal rate of current activation. In addition, after the flash, current decay slows because of the removal of nifedipine from the bath: open channel block is no longer evident. At longer times during a voltage step, the flash is less effective at recovering current amplitude, as shown in *B* and *C*. When the flash is delivered 50 (*B*) or 80 (*C*) ms into the step, blockade of both open and closed channels is relieved and there appear to be two components to the recovery.



As evident from the simulations of  $Ba^{2+}$  currents and the effects of flashes presented at various times during the current waveform, the modulated receptor model can provide a fairly accurate representation of our experimental observations with nifedipine.

This work was supported by the Del E. Webb Foundation (fellowship to A.M.G.), Fulbright-Hayes (travel grant to A.M.G.), the Los Angeles Affiliate of the American Heart Association (fellowships to A.M.G. and J.M.N.), the National Office of the American Heart Association (established investigatorship to J.M.N.), and the National Institutes of Health (grant GM-29836).

*Original version received 4 December 1984 and accepted version received 7 May 1985.*

#### REFERENCES

- Adams, P. R. 1976. Drug blockade of open end-plate channels. *J. Physiol. (Lond.)* 260:531–552.
- Armstrong, C. M. 1966. Time course of TEA<sup>+</sup>-induced anomalous rectification in squid giant axons. *J. Gen. Physiol.* 50:491–503.
- Bean, B. P. 1984. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA* 81:6388–6392.
- Bean, B. P., C. J. Cohen, and R. W. Tsien. 1983. Lidocaine block of cardiac sodium channels. *J. Gen. Physiol.* 81:613–642.
- Bean, B. P., M. C. Nowicky, and R. W. Tsien. 1984.  $\beta$ -adrenergic modulation of the number of functional calcium channels in frog ventricular heart cells. *Nature (Lond.)* 307:371–375.
- Bevington, P. R. 1969. *Data Reduction and Error Analysis for the Physical Sciences*. McGraw-Hill Book Co., New York. 204–246.
- Cachelin, A. B., J. E. dePeyer, S. Kokubun, and H. Reuter. 1983. Calcium channel modulation by 8-bromo-cyclic AMP in cultured heart cells. *Nature (Lond.)* 304:462–464.
- Cavalie, A., R. Ochi, D. Pelzer, and W. Trautwein. 1983. Elementary currents through  $Ca^{2+}$  channels in guinea pig myocytes. *Pflügers Arch. Ges. Physiol.* 398:284–297.
- DePover, A., M. A. Matlib, S. W. Lee, G. P. Dube, G. Grupp, and A. Schwartz. 1982. Specific binding of [<sup>3</sup>H]-nitrendipine to membranes from coronary arteries and heart in relation to pharmacological effects. Paradoxical stimulation by diltiazem. *Biochem. Biophys. Res. Commun.* 108:110–117.
- Ebel, V. S., H. Schutz, and A. Hornitschek. 1978. Untersuchungen zur analytik von nifedipin unter besonderer berucksichtigung der bei lichtexposition entstehenden umwandlungsprodukte. *Arzneim. Forsch.* 28:2188–2193.
- Ehara, T., and R. Kaufmann. 1978. The voltage- and time-dependent effects of (–)-verapamil on the slow inward current in isolated cat ventricular myocardium. *J. Pharmacol. Exp. Ther.* 207:49–55.
- Fenwick, E. M., A. Marty, and E. Neher. 1982. Sodium and calcium channels in bovine chromaffin cells. *J. Physiol. (Lond.)* 331:599–635.
- Flaim, S. F., and R. Zelis. 1981. Clinical use of calcium entry blockers. *Fed. Proc.* 40:2877–2881.
- Fleckenstein, A. 1977. Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* 17:149–166.
- Fleckenstein, A. 1983. History of calcium antagonists. *Circ. Res. (Suppl. I)* 52:3–16.
- Gurney, A. M., J. M. Nerbonne, and H. A. Lester. 1984. Photoremoval of nifedipine reveals the likely mechanism of action of this  $Ca^{++}$  antagonist. *Biophys. J.* 45:35a. (Abstr.)

- Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* 391:85–100.
- Hess, P., J. B. Lansman, and R. W. Tsien. 1984. Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature (Lond.)*. 311:538–544.
- Hille, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.
- Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)*. 117:500–544.
- Holck, M., S. Thorens, and G. Hausler. 1983. Does [<sup>3</sup>H]-nifedipine label the calcium channel in rat myocardium? *J. Receptor Res.* 3:191–198.
- Hondeghem, L. M., and B. G. Katzung. 1977. Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta.* 472:373–398.
- Hondeghem, L. M., and B. G. Katzung. 1984. Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annu. Rev. Pharmacol. Toxicol.* 24:387–423.
- Janis, R. A., and D. J. Triggle. 1983. New developments in Ca<sup>++</sup> channel antagonists. *J. Med. Chem.* 26:775–785.
- Kanaya, S., P. Arlock, B. G. Katzung, and L. M. Hondeghem. 1983. Diltiazem and verapamil preferentially block inactivated cardiac calcium channels. *J. Mol. Cell. Cardiol.* 15:145–148.
- Kass, R. S. 1983. Measurement and block of voltage-dependent calcium current in the heart. A comparison of the actions of D600 and nisoldipine. In *Calcium Entry Blockers, Adenosine and Neurohumors*. G. F. Merrill and H. R. Weiss, editors. Urban & Schwarzenberg, Baltimore, MD. 1–19.
- Kass, R. S., and R. W. Tsien. 1975. Multiple effects of calcium antagonists on plateau currents on cardiac Purkinje fibers. *J. Gen. Physiol.* 66:169–192.
- Kegel, D. R., B. D. Wolf, R. E. Sheridan, and H. A. Lester. 1985. Software for electrophysiological experiments with a personal computer. *J. Neurosci. Methods.* 12:317–330.
- Kostyuk, P. G. 1981. Calcium channels in the neuronal membrane. *Biochim. Biophys. Acta.* 650:128–150.
- Lee, K. S., and R. W. Tsien. 1983. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature (Lond.)*. 302:790–794.
- Lux, H. D., and A. M. Brown. 1984. Single channel studies on inactivation of Ca<sup>++</sup> currents. *Science (Wash. DC)*. 225:432–434.
- Marquardt, D. W. 1963. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* 11:431–441.
- McDonald, T. F., D. Pelzer, and W. Trautwein. 1980. On the mechanism of slow calcium channel block in heart. *Pflügers Arch. Eur. J. Physiol.* 385:175–179.
- Morad, M., Y. E. Goldman, and D. R. Trentham. 1983. Rapid photochemical inactivation of Ca<sup>++</sup>-antagonists shows that Ca<sup>++</sup> entry directly activates contraction in frog heart. *Nature (Lond.)*. 304:635–638.
- Murphy, K. M. M., R. J. Gould, B. L. Largent, and S. H. Snyder. 1983. A unitary mechanism of calcium antagonist drug action. *Proc. Natl. Acad. Sci. USA.* 80:860–864.
- Nargeot, J., H. A. Lester, N. J. M. Birdsall, J. Stockton, N. H. Wassermann, and B. F. Erlanger. 1982. A photoisomerizable muscarinic antagonist. Studies of binding and conductance relaxations in frog heart. *J. Gen. Physiol.* 79:657–678.

- Nargeot, J., J. M. Nerbonne, J. Engels, and H. A. Lester. 1983. Time course of the increase in the myocardial slow inward current after a photochemically generated concentration jump of intracellular cAMP. *Proc. Natl. Acad. Sci. USA.* 80:2395-2399.
- Nayler, W. G. 1983. The heterogeneity of the slow channel blockers (calcium antagonists). *Int. J. Cardiol.* 3:391-400.
- Nerbonne, J. M., S. Richard, and J. Nargeot. 1985.  $\text{Ca}^{++}$  channels are unblocked within a few milliseconds after photoconversion of nifedipine. *J. Mol. Cell. Cardiol.* 17:511-515.
- Noble, S., and S. Yahkin. 1981. The calcium and frequency dependence of the slow inward current "staircase" in frog atrium. *J. Physiol. (Lond.).* 310:57-75.
- Reuter, H., A. B. Cachelin, J. E. dePeyer, and S. Kokubun. 1983. Modulation of calcium channels in cultured cardiac cells by isoproterenol and 8-bromo-cAMP. *Cold Spring Harbor Symp. Quant. Biol.* 48:193-200.
- Reuter, H., and H. Scholz. 1977. A study of the ion selectivity and the kinetic properties of the calcium dependent slow inward current in mammalian cardiac muscle. *J. Physiol. (Lond.).* 264:17-47.
- Reuter, H., C. F. Stevens, R. W. Tsien, and G. Yellen. 1982. Properties of single calcium channels in cardiac cell culture. *Nature (Lond.).* 297:501-504.
- Sanguinetti, M. C., and R. S. Kass. 1984a. Photoalteration of calcium channel blockade in the cardiac Purkinje fiber. *Biophys. J.* 45:873-880.
- Sanguinetti, M. C., and R. S. Kass. 1984b. Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.* 55:336-348.
- Smith, R. D. 1983. Calcium entry blockers: key issues. *Fed. Proc.* 42:201-207.
- Strichartz, G. R. 1973. The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *J. Gen. Physiol.* 62:37-57.
- Uehara, A., and J. R. Hume. 1984. Interactions of organic Ca channel antagonists with Ca channels in isolated frog atrial cells: test of a modulated receptor hypothesis. *Biophys. J.* 45:50a. (Abstr.)
- Yamamura, H. I., H. Schoemaker, R. G. Boles, and W. R. Roeske. 1982. Diltiazem enhancement of [ $^3\text{H}$ ]-nitrendipine binding to calcium channel associated drug receptor sites in rat brain synaptosomes. *Biochem. Biophys. Res. Commun.* 108:640-646.